

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

## DESCRIPTION

RECOMBINANT HUMAN SM-11044-BINDING RECEPTOR PROTEINS  
EXHIBITING LIGAND-BINDING ACTIVITIES, AND THEIR USES

5

## TECHNICAL FIELD

The present invention relates to recombinant human SM-11044 ((L)-threo-3-(3,4-dihydroxyphenyl)-N-[3-(4-fluorophenyl)propyl] serine pyrrolidine amide hydrobromide)-binding receptor proteins exhibiting ligand-binding activities (abbreviated as SMBP hereinafter ), and their uses. More particularly, it relates to transformed cells that are designed to express a recombinant human SMBP at an elevated level to the extent that its ligand-binding activity can be measured by deleting the polythymidine sequence from the base sequence of the 3'-untranslated region, and cellular membrane fractions thereof, to recombinant human SMBPs isolated from the transformed cells or the cellular membrane fractions thereof, to a screening system for discovering human SMBP agonists/antagonists characterized by utilizing the transformed cells, the cellular membrane fractions thereof or the isolated recombinant human SMBPs, and to human SMBP agonists or antagonists obtainable by the screening system.

15  
20

## BACKGROUND ART

SM-11044 ((L)-threo-3-(3,4-dihydroxyphenyl)-N-[3-(4-fluorophenyl)propyl] serine pyrrolidine amide hydrobromide)-binding receptor protein (SMBP) was discovered as a new protein that is bound

25

by SM-11044, which is an agonist for  $\beta$ -adrenergic receptors, and by  
iodocyanopindolol, which is an antagonist for  $\beta$ -adrenergic receptors  
(Sugasawa, T. et al., J. Biol. Chem., 267, 21244-21252 (1997)). SMBP  
is a membrane protein resided at lung, ileum, and eosinophil  
5 membrane, and is believed to act as a receptor for SM-11044. SM-  
11044 was known to have activities to down-regulate the  
depolarization-mediated contraction of intestine and to inhibit migration  
of eosinophils, and has been believed to exert such SM-11044's  
functions via SMBP (Sugasawa, T. et al., J. Biol. Chem., 267, 21244-  
10 21252 (1997)).

Although the cDNA of a human SMBP was recently cloned  
(International Publication No. WO 98/26065), it was not reported that  
SM-11044 binds to any recombinant protein translated from the  
cDNA. In other words, there has been no report showing that a human  
15 SMBP is expressed at an elevated level to the extent that its ligand-  
binding activity can be measured, and, therefore, any SMBP has not yet  
been established in its particular availability.

#### DISCLOSURE OF THE INVENTION

20 The present invention aims to provide a recombinant human  
SMBP exhibiting ligand-binding activities, and its use. More  
particularly, it aims to provide transformed cells that are designed to  
express a recombinant human SMBP at an elevated level to the extent  
that its ligand-binding activity can be measured by deleting the  
25 polythymidine sequence from the base sequence of the 3'-untranslated  
region, cellular membrane fractions thereof, and recombinant human

SMBPs isolated from the transformed cells or the cellular membrane fractions thereof, as well as a screening system for discovering human SMBP agonists/antagonists characterized by utilizing the transformed cells, the cellular membrane fractions thereof or the isolated  
5 recombinant human SMBPs, and human SMBP agonists or antagonists obtainable by the screening system.

As mentioned above, SMBP has been believed to be a receptor that mediates actions to down-regulate the depolarization-mediated contraction of intestine and to inhibit migration of eosinophils.

10 Accordingly, it is expected that substances exhibiting an agonistic activity for SMBP would bind to SMBP to exert the functions as mentioned above, thereby leading to pharmaceutical compositions for treating inflammatory diseases involving eosinophil infiltration, asthma, or bowel diseases.

15 The present inventors attempted to construct a screening system for discovering efficiently ligands binding to SMBP, which comprises using SMBP in view of the development of such pharmaceutical compositions. International Publication No. WO 98/26065 describes that western blotting with use of anti-human  
20 SMBP antibody revealed that a SMBP protein was expressed by COS cells transformed with the recombinant human SMBP cDNA. However, the SMBP protein was expressed in a quite small amount, and, consequently, it has not been reported that any human SMBP is expressed in a sufficiently high level to construct screening systems.  
25 In fact, the inventors obtained the relevant human SMBP cDNA fragment (SEQ ID NO: 1) from the applicant of the International

Application (WO 98/26065), Vetigen, and transformed the cDNA into COS-1 cells or CHO-K1 cells. Then, the inventors determined a ligand-binding activity of the transfectants, but found no activity. Due to these facts, the inventors believed either of 1) that any protein had not translated from the human SMBP cDNA, or 2) that, even if a protein was translated, it had not been expressed at an elevated level to the extent that its ligand-binding activity can be measured.

The present inventors presumed that drawbacks involving the structure of the cDNA would cause no or little expression of the protein. Restudy of the base sequence of SEQ ID NO: 1 revealed that the 3'-untranslated region contains a polythymidine sequence consisting of a consecutive sequence of as many as 37 thymidines, and that the polyuridine sequence of the mRNA corresponding to the polythymidine sequence binds to the polyadenine tail (poly-A) residing at the 3'-terminus of the SMBP mRNA to form certain secondary structure, which would thereby restrain translation into proteins.

On the basis of the above presumption, by deleting the polythymidine sequence from the 3'-untranslated region in the base sequence of the human SMBP cDNA depicted in SEQ ID NO: 1, the inventors have successfully expressed a recombinant human SMBP at an elevated level to the extent that its ligand-binding activity can be measured for the first time. Further, the inventors have successfully established, for the first time, a screening system for discovering SMBP agonists/antagonists that are effective in a human, owing to the availability of such measurement of ligand-binding activity.

The present invention has been completed on the basis of the

findings as described above.

Thus, the present invention relates to:

(1) A process for expressing a recombinant protein at an elevated level, which comprises deleting a sequence comprising the polythymidine sequence from the base sequence of the 3'-untranslated region;

(2) A process for expressing a recombinant human SMBP at an elevated level, which comprises;

(a) preparing a DNA wherein a sequence comprising the polythymidine sequence is deleted from the 3'-untranslated region in the base sequence of SEQ ID NO: 1;

(b) introducing the DNA of the above (a) into an expression vector;

(c) transforming a host cell with the expression vector of the above (b); and

(d) culturing the transformed cells of the above (c) under an appropriate condition;

(3) A DNA encoding a recombinant human SMBP, which is characterized in that;

(e) a sequence comprising the polythymidine sequence is deleted from a 3'-region from position 1875 in the base sequence of SEQ ID NO: 1; and

(f) the recombinant human SMBP that is a translation product of the DNA can be expressed at an elevated level to the extent that its ligand-binding activity can be measured;

(4) The DNA of the above (3) wherein a sequence comprising all or part of the base sequence from positions 1899 to 1935 of SEQ ID NO: 1 is deleted;

(5) The DNA of the above (4) wherein the portion of the base sequence from positions 1875 to 2072 of SEQ ID NO: 1 is deleted.

(6) The DNA of the above (5), which consists of the base sequence of SEQ ID NO: 3.

5 (7) An expression vector which carries the DNA of any one of the above (3) to (6).

10 (8) A transformed cell expressing a recombinant protein at an elevated level to the extent that its ligand-binding activity can be measured, which is obtainable by the process of the above (1) or (2), or a cellular membrane fraction thereof.

(9) A transformed cell expressing a recombinant human SMBP at an elevated level to the extent that its ligand-binding activity can be measured, which is obtainable by the process of the above (2), or a cellular membrane fraction thereof.

15 (10) The transformed cell of the above (9), which is obtainable by culturing cells transformed with the expression vector of the above (7) under an appropriate condition, or a cellular membrane fraction thereof.

20 (11) A process for preparing a recombinant human SMBP, which comprises isolating the recombinant human SMBP from the transformed cells or the cellular membrane fractions thereof according to the above (9) or (10).

(12) A recombinant human SMBP obtainable by the process of the above (11).

25 (13) A screening system for discovering a human SMBP agonist or antagonist, which comprises utilizing the transformed cell or the

cellular membrane fraction thereof according to the above (9) or (10), or the recombinant human SMBP of the above (12).

(14) A human SMBP agonist or antagonist obtainable by the screening system of the above (13).

5 (15) A pharmaceutical composition for inhibiting migration of eosinophils, or for relaxing the contraction of intestine, which comprises the agonist of the above (14).

According to the present invention, a recombinant human SMBP has been successfully expressed at an elevated level to the extent  
10 that its ligand-binding activity can be measured, by deleting the polythymidine sequence from the 3'-untranslated region of the DNA encoding the human SMBP. It is understood that this would result from the consequence of the right translation into a protein provided by  
15 deletion of the polythymidine sequence that restrains the translation into a protein due to certain secondary structure formed by binding the polyuridine sequence in the mRNA corresponding to the polythymidine sequence to the polyadenine tail (poly-A) residing at the 3'-terminus of the mRNA.

Besides the human SMBP DNA as shown above, it is believed  
20 that similar effects can be also obtained by deleting a polythymidine sequence in cases of DNAs of other recombinant proteins having a polythymidine sequence in the 3'-regions. Accordingly, the present invention provides a process for expressing recombinant proteins in  
25 general at elevated levels, as well as transformed cells expressing recombinant proteins obtainable by the process, and the cellular membrane fractions thereof.



Particular steps of the process for conducting the elevated expression as mentioned above are provided below, taking a recombinant human SMBP for instance. Thus, the process for expressing a recombinant human SMBP at an elevated level comprises;

- (a) preparing a DNA wherein a sequence comprising the polythymidine sequence is deleted from the 3'-untranslated region in the base sequence of SEQ ID NO: 1;
- (b) introducing the DNA of the above (a) into an expression vector;
- (c) transforming a host cell with the expression vector of the above (b); and
- (d) culturing the transformed cells of the above (c) under an appropriate condition. Details of each step of these (a) to (d) are described hereinafter.

In the present invention, the term "DNA" refers to any DNA as long as the DNA encodes a recombinant human SMBP, of which the polythymidine sequence is deleted from the 3'-untranslated region in the base sequence of SEQ ID NO: 1. Specific examples include a DNA encoding a recombinant human SMBP, which is characterized in that;

- (a) a sequence comprising the polythymidine sequence is deleted from a 3' region from position 1875 in the base sequence of SEQ ID NO: 1; and, as a consequence of the deletion, (b) the translation product of the DNA, the recombinant human SMBP, may be expressed at an elevated level to the extent that its ligand-binding activity can be measured.

In this context, "a DNA encoding human SMBP" may be readily cloned on the basis of the base sequence of human SMBP described in

WO 98/26065 by using as PCR primers or probes for hybridization an appropriate portion in the base sequence according to conventional methods (consulting a standard text such as "Molecular Cloning", 2nd ed., Cold Spring Harbor Laboratory Press (1989)). Further, alterations  
5 such as substitution, deletion, or addition may be also made to the cloned DNA according to "Molecular Cloning" 2nd Edt. Chapter 15, Cold Spring Harbor Laboratory Press (1989), and such altered SMBPs-encoding DNAs fall within the scope of the DNA encoding a recombinant human SMBP of the present invention as long as the expressed  
10 products of the DNAs, altered SMBPs, exhibit a binding activity to ligands such as SM-11044.

Among these DNAs encoding recombinant human SMBPs, specific examples of the present invention include a DNA which is characterized in that; (a) a sequence comprising the polythymidine  
15 sequence is deleted from a 3' region from position 1875 in the base sequence of SEQ ID NO: 1; and (b) the translation product of the DNA, the recombinant human SMBP, may be expressed at an elevated level to the extent that its ligand-binding activity can be measured.

In this connection, with respect to the base sequence up to  
20 position 1875 in the sequence of human SMBP-DNA of SEQ ID NO: 1, the base sequence up to position 1875 in the sequence of SEQ ID NO: 1, or a sequence wherein the sequence contains the above alteration in said base sequence, and the expressed product of the DNA, a recombinant SMBP, exhibits a binding activity to ligands such as SM-  
25 11044 are fallen within the scope of the present invention. Specific examples include the sequence up to position 1874 in SEQ ID NO: 1,

the sequence up to position 1827 in SEQ ID NO: 3, and the like.

With respect to a 3' region from position 1875 in the base sequence of SEQ ID NO: 1, any DNA wherein a sequence comprising "polythymidine sequence" expected to inhibit the expression of a human SMBP protein is deleted falls within the scope of the present invention. Methods for deleting a sequence comprising the polythymidine sequence include a method for the deletion wherein suitable restriction enzyme sites positioned at each side of the polythymidine are utilized if any, and a method for the deletion involving well-known techniques such as PCR (Molecular Cloning: A Laboratory Manual 2nd Edt. Chapters 1-3, Cold Spring Harbor Laboratory Press (1989)).

In this connection, the term "polythymidine sequence" refers to a sequence comprising consecutive thymidines, of which the deletion leads to the expression of the translation product, the recombinant human SMBP, at an elevated level to the extent that its ligand-binding activity can be measured.

Examples of the method for measuring a ligand-binding activity. include the method described in *J. Biol. Chem.*, 267, 21244-21252 (1997). The method in principle comprises determining a binding reactivity to 1nM [<sup>125</sup>I]-iodocyanopindolol used as a ligand, determining a nonspecific binding reactivity of iodocyanopindolol by use of 10<sup>-4</sup> M SM-11044, and subtracting the nonspecific binding reactivity from the binding reactivity so as to measure a ligand-binding activity of SMBP protein (*J. Biol. Chem.*, 267, 21244-21252 (1997)).

Specifically, a human SMBP expression vector is prepared by

introducing a candidate DNA for the DNA of the present invention into an expression vector, and a transformed cell is prepared by introducing the SMBP expression vector into a host cell. Then, the resultant transformed cells or cellular membrane fractions thereof are subjected to the system for measuring a ligand-binding activity as shown above (the expression vector, the transformed cells, and the cellular membrane fractions thereof are further described hereinafter). Examples of the method for measuring a ligand-binding activity include the substantially same method as that described in *J. Biol. Chem.*, 267, 21244-21252 (1997) mentioned above, and a method that is detailed in Example 6. Specifically, a 96-well Multiscreen plate (Millipore) in which a piece of glass fiber paper is placed on the bottom of the wells is treated with Tris-HCl buffered saline containing 0.3% polyethyleneimine (Sigma) (reconstituted to pH7.4 with 6N HCl), and washed by vacuum filtration with Tris-HCl buffered saline (pretreatment). Then, 200  $\mu$ l of Tris-HCl buffered saline containing 1nM [ $^{125}$ I]-iodocyanopindolol (Amersham) and a cellular membrane fraction as mentioned above (50  $\mu$ g of membrane protein) that have been incubated at 37 °C for 30 minutes is added to each well on the 96-well Multiscreen plate, and are washed by vacuum filtration. The cellular membrane fraction is harvested on the glass fiber paper, and washed by vacuum filtration with 200  $\mu$ l of an ice-cooled Tris-HCl buffered saline. Then, the amount of [ $^{125}$ I]-iodocyanopindolol bound to the membrane fraction trapped on the paper is determined by a gamma counter to represent a total binding. Nonspecific binding of [ $^{125}$ I]-iodocyanopindolol is determined by conducting an incubation as mentioned above in the

presence of  $10^{-4}$  M SM-11044 (Sumitomo Pharmaceuticals Co., Ltd. it can be prepared according to the process described in Japanese Patent Publication (kokai) No. 132935/1985, Japanese Patent Publication (kokoku) No. 50499/1993) and then conducting similar procedures to those mentioned above. A ligand-binding to SMBP may be calculated by subtracting the nonspecific binding from the total binding.

DNAs of the present invention can be readily selected by subjecting transformed cells introduced with a candidate DNA for the DNA of the present invention, or cellular membrane fractions thereof to the system for measuring a ligand-binding activity as shown above. The system for the measurement may be appropriately modified as far as the common knowledge of those skilled in the art. For example, SM-11044, BRL-35135A (Smith Kline Beecham), or alprenolol (Ciba Geigy) labeled with [ $^{125}$ I] or [ $^3$ H] may be used instead of [ $^{125}$ I]-iodocyanopindolol.

Among the DNAs of the present invention, suitable examples include a DNA wherein a sequence comprising all or part of the polythymidine sequence residing from positions 1899 to 1935 is deleted from the 3'-untranslated region in the base sequence of SEQ ID NO: 1. In this connection, "the part" is preferably about 30 bases in length since it should have been bound to the polyadenine tail (poly-A) attached to the 3'-terminus of the SMBP mRNA to form certain secondary structure, although any length in the part may be acceptable as long as a recombinant human SMBP can be expressed at an elevated level to the extent that its ligand-binding activity can be measured.

More suitable examples of the DNA of the present invention includes a DNA wherein the sequence from positions 1875 to 2072 is

deleted from the base sequence of SEQ ID NO: 1, and even more suitable examples include the human SMBP DNA consisting of the base sequence of SEQ ID NO: 3.

5 The DNA of the present invention as mentioned above may be incorporated into an expression vector according to conventional methods to obtain a SMBP expression vector carrying the DNA of the invention.

10 In this connection, the expression vectors to be incorporated with the DNA of the present invention may be any vector capable of expressing efficiently the subject in a host cell, and preferably include a pcDNA3.1 derivative, pRc/RSV, pRc/CMV, a pEF derivative (all of them are from Invitrogen), pIRESneo (Clontech), and a pREP9 derivative (Invitrogen).

15 The SMBP expression vector thus prepared may be transformed into host cells to prepare transformed cells wherein the expression vector of the present invention is retained stably in chromosomes of the host cells. In this connection, host cells may be any cell as long as a foreign gene may be stably integrated into chromosomes of the host cells, and mammal cells are preferred. Examples of the host cells  
20 include CHO cells, L929 cells, C127 cells, and BALB/c3T3 cells as well as variants thereof wherein a dihydrofolate reductase or thymidine kinase function is defective.

25 Examples of methods for transforming the expression vector of the present invention into host cells include the calcium phosphate method (*J. Virol.*, 52, 456-467 (1973)), a method involving LT-1 (Panvera), and a method involving lipids for gene-introduction (Lipofectamine,

Lipofectin; Gibco-BRL). After the transformation, the cells may be cultured in a conventional medium containing a selective marker (for example, Zeocin in the case of using pcDNA3.1/Zeo(+) shown above as an expression vector) to select the transformed cells wherein the expression vector of the present invention is stably retained in chromosomes of the host cells.

The transformed cells thus obtained may be continuously cultured under an appropriate condition to prepare transformed cells of the present invention wherein a recombinant human SMBP is expressed on the cellular membrane at an elevated level to the extent that its ligand-binding activity can be measured.

The term "appropriate condition" refers to a condition wherein a cultivation is conducted at 37 °C under 5%CO<sub>2</sub> in a culture medium suitable for respective host cell, and examples include a condition wherein CHO cells are cultured at 37 °C under 5%CO<sub>2</sub> in HAM'S F-12 medium containing 10% bovine calf serum.

Cellular membrane fractions of the present invention can be prepared from the transformed cells thus obtained wherein a human SMBP is expressed at an elevated level. For example, a process for preparing the cellular membrane fractions may comprise adding to the cells a hypotonic homogenate buffer (10 mM Tris-HCl buffer, 1 mM EDTA, 0.5 mM PMSF or 1mM AEBSF, 5 µg/ml aprotinin, 5 µg/ml leupeptin, pH7.4), allowing the mixture to stand at 4 °C for about 30 minutes so as to destroy the cells due to the hypotonic condition, homogenizing it by the pipetting, and centrifuging the homogenate at 4 °C at 50000xg for about 30 minutes, thereby obtaining the cellular

membrane fractions of the present invention.

Alternatively, for example, the method described by F. Pietri-Rouxel et al. (*Eur. J. Biochem.*, 247, 1174-1179 (1997)) may be used to prepare the cellular membrane fractions of the present invention.

5 A recombinant human SMBP of the present invention can be isolated from the transformed cells of the cellular membrane fractions of the present invention thereof thus obtained. Specifically, for example, the method of R. G. Shorr, et al. (*Proc. Natl. Acad. Sci. USA*, 79, 2778-2782 (1982); *J. Biol. Chem.* 257, 12341-12350 (1982)) may be used to  
10 obtain a crude extract of a human SMBP of the present invention. Further, a method for purifying a human SMBP from the crude extract is exemplified by the method of J. L. Benovic, et al. (*Biochem.*, 23, 4510-4518 (1984)).

Specific examples of the recombinant human SMBP of the  
15 present invention include a human SMBP consisting of the amino acid of SEQ ID NO: 2 or SEQ ID NO: 4, and a altered SMBP consisting of said amino acid that contains substitution, deletion, and/or addition is also fallen within the scope of the recombinant human SMBPs of the present invention as long as the latter has a binding activity to ligands  
20 such as SM-11044.

Agonists or antagonists binding to the human SMBP can be screened by use of either of the transformed cells expressing the recombinant human SMBP at an elevated level, the cellular membrane fractions thereof, or the isolated recombinant human SMBP, each of  
25 which are as obtained above.

The screening by use of the cellular membrane fractions of the



transformed cells expressing the recombinant human SMBP at an elevated level may be conducted for example by the following method.

First of all, a Tris-HCl buffered saline containing the cellular membrane fraction of the present invention (50-200  $\mu$ g membrane protein) and 1nM [ $^{125}$ I]-iodocyanopindolol is incubated at 37 °C for 30 minutes, and the reaction is added to each well on a 96 well-

Multiscreen plate that has been treated by a similar pretreatment to that in "the method for measuring a ligand-binding activity" as mentioned above, then being aspirated by vacuum filtration.

Subsequently, a similar treatment to that in "the method for measuring a ligand-binding activity" as mentioned above is conducted, and the amount of [ $^{125}$ I]-iodocyanopindolol bound to the membrane fraction trapped on the paper is determined by a gamma counter to give a binding, which represents binding A. Then, the incubation as shown

above is conducted in the presence of a test compound at a normal range of concentrations ( $10^{-12}$ - $10^{-4}$ M), and then a similar procedure is conducted to give a binding, which represents binding B. A binding that is provided by use of  $10^{-4}$ M SM-11044 instead of a test compound

represents binding C. Accordingly, when the value subtracted binding

B from binding A is equivalent to one subtracted binding C from binding A, the test compound is estimated to have 100% SMBP-binding activity, and, when the value is the half, the compound is estimated to have the 50%. SMBP ligands thus selected are subjected to an assay as

described in either *J. Biol. Chem.*, 267, 21244-21252 (1997),

*Eur.J.Pharmacol.* 216,207-215 (1992), or *Agents Actions* 37, 233-237

(1992). At that time, when down-regulating the contraction of intestine

or inhibiting the migration of eosinophils equivalently to or more than SM-11044, the ligands may be a SMBP agonist, whereas when showing the inverse activities, they may be a SMBP antagonist.

The screening by use of the transformed cells expressing the human SMBP of the present invention at an elevated level may be conducted for example by the following method.

First of all, the transformed cells expressing the human SMBP of the present invention at an elevated level that have been washed with Dulbecco's phosphate buffered saline (Gibco) are incubated with 1-5 mM EDTA-Dulbecco's phosphate buffered saline at room temperature, and then removed from the culture dish. After centrifuged (400 x g, 10 min, 4 °C) and the supernatant being removed by aspiration, the cells are suspended by the pipetting in Tris-HCl buffered saline containing 0.2% bovine serum albumin. The cells at  $5 \times 10^5$ , 1nM [ $^{125}$ I]-iodocyanopindolol, and Tris-HCl buffered saline containing 0.2% bovine serum albumin are incubated at 37°C for 30 minutes, and the reaction is added to each well on a 96 well-Multiscreen plate that has been treated by a similar pretreatment to that in "the method for measuring a ligand-binding activity" as mentioned above, the being aspirated by vacuum filtration. Subsequently, a similar treatment to that that in "the method for measuring a ligand-binding activity" as mentioned above is conducted, and the amount of [ $^{125}$ I]-iodocyanopindolol bound to the membrane fraction trapped on the paper is determined by a gamma counter to give a binding, which represents binding A. Then, incubation as shown above is conducted in the presence of a test compound at a normal rage of concentrations ( $10^{-12}$ - $10^{-4}$ M), and a

similar procedure is conducted to give a binding, which represents binding B. A binding that is provided by use of  $10^{-4}$ M SM-11044 instead of a test compound represents binding C. Subsequent procedures for the estimation are as shown above.

5           The screening by use of the recombinant human SMBP isolated from the transformed cells or the cellular membrane fractions thereof according to the present invention may be conducted for example by the following method.

          First of all, an isolated SMBP standard is prepared by  
10       solubilizing the transformed cells or the cellular membrane fractions thereof by use of a suitable solubilizer, preferably,  $\beta$ -D-octylglucoside. Two hundred fifty  $\mu$ l of Tris-HCl buffered saline containing the isolated SMBP standard and 1nM [ $^{125}$ I]-iodocyanopindolol is incubated at 37 °C for 30 minutes, and then 250  $\mu$ l of an ice-cooled Tris-HCl buffered  
15       saline is added thereto, thereby quenching the reaction. The reaction is applied to Sephadex G-50 column (Pharmacia) that have been equilibrated with Tris-HCl buffered saline, and eluted with Tris-HCl buffered saline. The amount of [ $^{125}$ I]-iodocyanopindolol bound to the  
20       SMBP fraction thus isolated by the gel filtration is determined by a gamma counter to give a binding, which represents binding A. Subsequently, incubation as shown above is conducted in the presence of a test compound at a normal range of concentrations ( $10^{-12}$ - $10^{-4}$ M), and a similar procedure is conducted to give a binding, which represents  
25       binding B. A binding that is provided by use of  $10^{-4}$ M SM-11044 instead of a test compound represents binding C. Subsequent procedures for the estimation are as shown above.

The screening systems as described above can be modified as appropriate within the knowledge of those skilled in the art.

Human SMBP agonists obtainable by the above screening systems are useful for an agent for inhibiting migration of eosinophils, or relaxing the contraction of intestine, and, specifically, are useful as a medicament or prophylactic for inflammatory diseases, asthma, or bowel diseases such as allergic gastrointestinal symptoms, involving eosinophil infiltration.

Agonists or antagonists obtainable by the screening systems of the present invention may be used for a pharmaceutical composition as shown above in accordance with conventional manners. For example, such substances can be administered orally in the form employed commonly in the art, such as tablets, capsules, syrups, suspensions, or the like. Such substances can also be administered parenterally in the form of, for example, solutions, emulsions, suspensions, or the like, as well as can be administered by rectal route in the form of suppositories. Such suitable dosage forms may be prepared in accordance with conventional manners by combining the active ingredient with conventional carriers, excipients, binders, stabilizing agents, or the like. Injectable formulations can additionally contain buffers, solubilizing agents, or isotonicizing agents. Doses and frequencies vary depending on, for example, the disease and condition to be treated, the age and weight of a particular patient, a dosage form, and the like, and a typical daily dose for adults of active ingredients in case of oral formulations may range about 1 mg to about 1000 mg, preferably, about 10 to about 500 mg, which may be administered at a

time or in portions. In case of the injectable formulations, a dose of active ingredients may range about 0.1 mg to about 500 mg, preferably, about 3 mg to about 100 mg, which may be administered at a time or in portions.

5

#### BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a graph showing the values of the ligand-binding activities that were measured using the cellular membranes of COS-7 cells transfected with pcDNA3.1/Zeo(+), SMBP-pcDNA3.1/Zeo(+),  
10 SMBP-Kozak-pcDNA3.1/Zeo(+), or SMBP-Kozak-pcDNA3.1/Zeo(+) poly-T free, wherein these are indicated by CHO-pcDNA3.1, CHO-SMBP, CHO-SMBP-Kozak, and CHO-SMBP-Kozak-poly T free, respectively. The results represent means  $\pm$  standard error of the duplicate experiments. The axis of ordinates indicates the amounts (CPM) of  
15 [<sup>125</sup>I]-iodocyanopindolol bound specifically to the SMBP. The symbols "\*\*\*", "+", and "N.S." mean that there is a significant difference ( $p < 0.01$ ) in relation to CHO-pcDNA3.1, that there is a significant difference ( $p < 0.05$ ) in relation to CHO-SMBP, and that there is no significant difference in relation to CHO-pcDNA3.1 or CHO-SMBP, respectively.

20

#### BEST MODE FOR CARRYING OUT THE INVENTION

The present invention is further illustrated by the following examples, but is not restricted by these examples in any way.

25

##### Example 1

Introduction of *Bam*HI/*Xba*I-cleaved fragment of SMBP-cDNA into

expression vector

The *Bam*HI/*Xba*I-cleaved fragment of SMBP-cDNA described in WO 98/26065 was obtained from the applicant of the patent application, Vetigen. The base sequence of the cDNA was sequenced by Takara Shuzo Co., Ltd. in accordance with a conventional method. The base sequence determined is shown in SEQ ID NO: 1, and the amino acid sequence of the protein encoded by the cDNA is shown in SEQ ID NO: 2. The base sequence of SEQ ID NO: 1 contains the base sequence disclosed as SEQ ID NO: 13 in WO 98/26065, and is further flanked by both 5' and 3' sequences.

Subsequently, the cDNA fragment was introduced into an expression vector, pcDNA3.1/Zeo(+) (Invitrogen), which had been cleaved with *Bam*HI and *Xba*I, to give an expression plasmid, SMBP-pcDNA3.1/Zeo(+). *E. coli* JM 109 (Toyobo Co. Ltd.) was transformed with the plasmid SMBP-pcDNA3.1/Zeo(+), and cultured overnight on a LB plate containing 50 µg/ml ampicillin to give transformants. Plasmids prepared from the transformants according to a conventional procedure were cleaved with *Bam*HI and *Xba*I to confirm that the SMBP-cDNA (about 2 kb) was inserted into the fragment. Further, cleavage with *Apa*I confirmed the orientation of the inserted sequence.

## Example 2

Binding of Kozak sequence to SMBP-cDNA fragment and introduction into expression vector

In surrounding sequences of ATG, a start codon, of the SMBP-cDNA fragment (at positions 49-51 in SEQ ID NO: 1), the Kozak's

consensus sequence (ACCATGG) presumed to be necessary to translate efficiently mRNAs into proteins is not found. Accordingly, the surrounding sequence of the start codon of the SMBP-cDNA fragment was replaced with ACCATGG as shown below in order to improve expression efficiency of proteins.

First, the following adapter containing the Kozak's consensus sequence and *HindIII*/*NotI* cleavage sites was prepared by annealing two types of different oligomers at 70 °C for 10 minutes:

Adapters containing the Kozak's sequence

5'-AGC TTC CAC CAT GGC-3'

3'-AG GTG GTA CCG CCG G-5'

The adapter thus prepared was incorporated into the expression vector pcDNA3.1/Zeo(+), which had been cleaved with *HindIII*/*NotI* to give a plasmid, Kozak-pcDNA3.1/Zeo(+). *E. coli* JM 109 was transformed with the plasmid Kozak-pcDNA3.1/Zeo(+), and cultured overnight on a LB plate containing 50 µg/ml ampicillin to give transformants. The multicloning sites in pcDNA3.1/Zeo(+) contain a *Bam*HI cleavage site between the *HindIII*-*NotI* sites, and, therefore, any plasmid without the inserted adapters would be cleaved with *Bam*HI. Thus, the presence of the inserted adapters was determined by whether or not plasmids prepared from the transformants could be cleaved with *Bam*HI.

Subsequently, the SMBP-cDNA fragment prepared by digesting the *Bam*HI/*Xba*I-cleaved fragment of the SMBP-cDNA with *NotI* was introduced into Kozak-pcDNA3.1/Zeo(+), which had been cleaved with *NotI*, to give an expression plasmid, SMBP-Kozak-pcDNA3.1/Zeo(+). *E.*

*coli* JM 109 (Toyobo Co. Ltd.) was transformed with the plasmid SMBP-Kozak-pcDNA3.1/Zeo(+), and cultured overnight on a LB plate containing 50 µg/ml ampicillin to give transformants. The plasmids prepared from the transformants were cleaved with *Hind*III and *Xba*I to confirm that the SMBP-cDNA (about 2 kb) was inserted into the plasmid. Further, cleavage with *Apa*I confirmed the orientation of the inserted sequence.

The amino acid sequence of the SMBP protein translated from the plasmid SMBP-Kozak-pcDNA3.1/Zeo(+) prepared as shown above was the sequence wherein the three amino acids at positions 2 to 4, His-Ala-Arg, is deleted from the amino acid sequence of SEQ ID NO: 2.

### Example 3

Deletion of polythymidine sequence from expression plasmid SMBP-Kozak-pcDNA3.1/Zeo(+), and reintroduction into expression vector pcDNA3.1/Zeo(+)

A polythymidine sequence consisting of a consecutive sequence of 37 thymidines (poly-T) exists downstream of the stop codon (TAG) in the SMBP-cDNA, which sequence is between positions 1899-1935 in the base sequence of SEQ ID NO: 1. The inventors presumed that the poly-U in the mRNA corresponding to the poly-T should bind to the polyadenine tail (poly-A) residing at the 3'-terminus of the SMBP-mRNA to form certain secondary structure, which would destabilize the m-RNA, or block its transport through the nuclear membrane, thereby restraining translation into proteins. Thus, the base sequence



downstream of position 1875 was excised from the base sequence of SEQ ID NO: 1 using restriction enzyme Ksp632I to give a SMBP-cDNA fragment wherein the portion of 198 bases containing the poly-T part was deleted, and the fragment was then introduced into an expression vector in accordance with the procedures as shown below.

Specifically, the expression plasmid SMBP-Kozak-pcDNA3.1/Zeo(+) as prepared in Example 2 was cleaved with Ksp632I to isolate a fragment (about 2.9 kb) wherein a SMBP-cDNA from which the portion of 198 bases containing the poly-T part was deleted, and a portion of pcDNA3.1/Zeo(+) were bound together. Then, the fragment was cleaved with *HindIII*, and a *HindIII*/Ksp632I-cleaved fragment of the SMBP-cDNA (about 1.8 kb) was isolated. The fragment was blunted with T4DNA polymerase (Takara Syuzo), and the blunt-ended fragment was introduced into an expression vector pcDNA3.1/Zeo(+) cleaved with *EcoRV* to give an expression plasmid SMBP-Kozak-pcDNA3.1/Zeo(+) poly-T free. The base sequence of the SMBP-cDNA carried on the expression plasmid SMBP-Kozak-pcDNA3.1/Zeo(+) poly-T free is shown in SEQ ID NO: 3, and the amino acid sequence thereof is shown in SEQ ID NO: 4.

*E. coli* JM 109 (Toyobo Co. Ltd.) was transformed with the plasmid SMBP-Kozak-pcDNA3.1/Zeo(+) poly-T free, and cultured overnight on a LB plate containing 50 µg/ml ampicillin to give transformants. The plasmids prepared from the transformants were cleaved with *HindIII*/*XbaI* to confirm that the SMBP-cDNA (about 2 kb) was inserted into the plasmid. Further, cleavage with *Apal* confirmed the orientation of the inserted sequence.

#### Example 4

##### Introduction of human SMBP-expression plasmid into animal cells

CHO-k1 cells were plated into wells of a 6-well plate at  $2 \times 10^5$  cells/well, and cultured for 24 hours in HAM's F-12 medium containing 10% fetal calf serum. The cells were transformed with pcDNA3.1/Zeo(+) (control), or each of human SMBP protein-expression plasmids, SMBP-pcDNA3.1/Zeo(+), SMBP-Kozak-pcDNA3.1/Zeo(+) and SMBP-Kozak-pcDNA3.1/Zeo(+) poly-T free as prepared in Examples 1 to 3, using lipids for gene incorporation (Lipofectamine; Gibco-BRL). From the fifth day after the transformations, the cells were cultured in HAM's F-12 medium containing 1.0 mg/ml Zeocin (Invitrogen) and 10% fetal calf serum, and cells wherein the plasmids were integrated in the chromosomes were selected.

#### Example 5

##### Preparation of CHO cellular membrane fractions containing human SMBP protein

CHO cells containing the human SMBP protein obtained in Example 4 were cultured in an adherent manner in a culture dish having a diameter of 10 cm, and washed with phosphate buffered saline (PBS). To the cells, 5 ml of an ice-cooled, hypotonic homogenate buffer (10 mM Tris-HCl buffer, 1 mM EDTA, 0.5 mM PMSF or 1mM AEBSF, 5  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml leupeptin, pH7.4) was added, and the mixture was allowed to stand at 4 °C for 30 minutes, thereby destroying the cells due to the hypotonic condition. The destroyed material was

homogenized by the pipetting, and the homogenate was centrifuged at 50000xg at 4 °C for about 30 minutes to give sediment of crude membrane fractions. The sediment was suspended in Tris-HCl buffered saline (Tris-HCl buffer, 154 mM sodium chloride, pH 7.4), and the suspension was stored at -80 °C. Each time used, a portion of this was thawed. Protein concentration was determined by a Protein Assay Kit (Bio-Rad) wherein bovine serum albumin is used as a standard.

#### Example 6

##### Measurement of ligand-binding activity of human SMBP protein

Sugasawa et al. (Sugasawa, T. et al., *J. Biol. Chem.*, 267, 21244-21252 (1997)) reported that a ligand-binding reactivity of a human SMBP protein can be measured by use of 1nM [<sup>125</sup>I]-iodocyanopindolol (2000 Ci/mmol; Amersham) as a ligand, and, specifically, the ligand-binding reactivity of a human SMBP protein can be estimated by determining a nonspecific binding reactivity of iodocyanopindolol by use of 10<sup>-4</sup> M SM-11044, and subtracting the nonspecific binding reactivity from the binding reactivity. According to the instructions of this literature, a ligand-binding activity of a SMBP protein was measured. Further, the binding assay was conducted using a 96-well microtiter plate in order to accelerate the assay.

First, a 96-well Multiscreen plate (Millipore) in which a piece of glass fiber paper was placed on the bottom of the wells was supplemented with Tris-HCl buffered saline containing 0.3% polyethyleneimine (Sigma) (reconstituted to pH7.4 with 6N HCl), treated for 30 minutes or more, and washed aspirating with Tris-HCl buffered

saline (pretreatment) by vacuum filtration.

Subsequently, 200  $\mu$ l of Tris-HCl buffered saline containing 1nM [ $^{125}$ I]-iodocyanopindolol and each cellular membrane fraction as prepared in Example 5 (50  $\mu$ g of membrane protein) that had been  
5 incubated at 37 °C for 30 minutes was added to each well on the 96-well Multiscreen plate, and were aspirated by vacuum filtration. The cellular membrane fractions were harvested on the glass fiber paper, and washed four times by vacuum filtration with 200  $\mu$ l of an ice-cooled Tris-HCl buffered saline. The amounts of [ $^{125}$ I]-iodocyanopindolol  
10 bound to the membrane fraction trapped on the paper were determined by a gamma counter to represent total bindings. Nonspecific bindings of [ $^{125}$ I]-iodocyanopindolol were determined by conducting incubations as mentioned above in the presence of  $10^{-4}$  M SM-11044, and then conducting similar procedures to those mentioned above. In each case,  
15 the nonspecific binding was subtracted from the total binding to give a ligand-binding to SMBP (a specific binding). The results are shown in Figure 1.

The membrane fraction of the CHO cells that were transformed with the expression plasmid introduced with the SMBP-cDNA described  
20 in WO 98/26065 (Example 1) (CHO-SMBP in Figure 1), and the membrane fraction of the CHO cells that were transformed with the expression plasmid introduced with the Kozak sequence (Example 2) (CHO-SMBP-Kozak in Figure 1) did not show any significant ligand-binding activities compared to the control (CHO-pcDNA3.1 in Figure  
25 1). Contrarily, the membrane fraction of the CHO cells that were transformed with the expression plasmid wherein the Kodak sequence

was introduced, and the poly-T sequence was deleted (Example 3) (CHO-SMBP-Kozak-poly T free in Figure 1) did show a significant ligand-binding activity compared to the control and to the CHO-SMBP cells before the deletion of the poly-T sequence. These results demonstrate that the deletion of the poly-T sequence causes an expression of a SMBP protein on the cellular membrane sufficient to show its ligand-binding activity.

#### Example 7

##### Screening for ligands of human SMBP protein

Two hundreds  $\mu$ l of Tris-HCl buffered saline containing 50  $\mu$ g of membrane protein of the cellular membrane fraction of the CHO cells transformed with SMBP-Kozak-pcDNA3.1/Zeo(+) poly-T free (as prepared in Example 5) and 1nM [ $^{125}$ I]-iodocyanopindolol is incubated at 37 °C for 30 minutes, and the reaction is added to each well on a 96 well-Multiscreen plate that has been treated by a similar pretreatment to that in Example 6, and aspirated by vacuum filtration. Then, a similar treatment to that that in Example 6 is conducted, and the amount of [ $^{125}$ I]- iodocyanopindolol bound to the membrane fraction trapped on the paper is determined by a gamma counter to give a binding, which represents binding A. Subsequently, the incubation as shown above is conducted in the presence of a test compound at a normal rage of concentrations ( $10^{-12}$ - $10^{-4}$ M), and then a similar procedure is conducted to give a binding, which represents binding B. A binding that is provided by use of  $10^{-4}$ M SM-11044 instead of a test compound represents binding C. When the value subtracted binding B

from binding A is equivalent to one subtracted binding C from binding A, the test compound is estimated to have 100% SMBP-binding activity, and, when the value is the half, the compound is estimated to have the 50%. SMBP ligands thus selected are subjected to an assay as described in either *J. Biol. Chem.*, 267, 21244-21252 (1997), *Eur.J.Pharmacol.* 216,207-215 (1992), or *Agents Actions* 37, 233-237 (1992). That procedure makes it possible to determine if the ligands have a SMBP-agonist activity, i.e., if the ligands down-regulate the contraction of intestine or if they inhibit the migration of eosinophils.

#### INDUSTRIAL APPLICABILITY

Cells that are transformed with an expression plasmid carrying a human SMBP cDNA wherein the polythymidine sequence is deleted according to the present invention can express a human SMBP to the extent that its ligand-binding activity can be measured. Accordingly, transformed cells containing a recombinant human SMBP, cellular membrane fractions thereof, or recombinant human SMBP isolated from said transformed cells or said cellular membrane fractions according to the present invention can be used to screen for ligands binding to a human SMBP. The screening systems of the present invention enable to efficiently discover human SMBP agonists or antagonists. Human tissues might be used in the screening system for discovering compounds binding to human SMBPs. However, when using human tissues, it is difficult to differentiate the intended ligands from those ligands to be bound to receptors other than the SMBPs existing at human tissues, such as human  $\beta 1$ -,  $\beta 2$ -, or  $\beta 3$ -adrenergic

receptors. The screening systems of the present invention make it possible to surely and efficiently select human SMBP agonists or antagonists, and, therefore, provides speedy development of pharmaceutical products such as pharmaceutical compositions for treating or preventing inflammatory diseases involving migration of eosinophils, antiasthmatic compositions, and pharmaceutical compositions for treating bowel diseases.